Tetrahedron Vol. 48, No. 23, pp. 5009-5014, 1992
Printed in Great Britain

0040-4020/92 $5.00+.00

Nitrogen Participation in the Deacylation of D-Glucosamine and α-Chymotrypsin Derivatives. Explanation of the Stereospecificity of Acyl-α-Chymotrypsin Hydrolysis

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(*Received in UK* 3 *January* 1992)

Dedicated to Professor Gábor Fodor on the occasion of his 75th birthday.

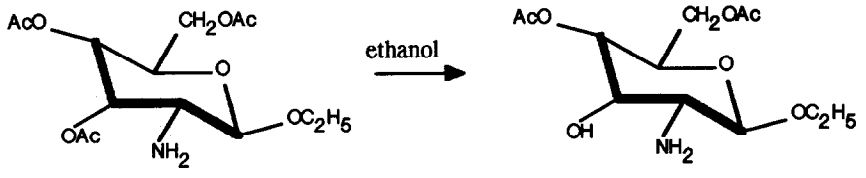
Selective deacylation, stereospecific ester hydrolysis, acylated derivatives of α-chymotrypsin, 2-amino-2-deoxy-D-glucose

Abstract:

The hydrolysis of ethyl 3,4,6-tri-O-acetyl-2-deoxy-2-amino-β-D-glucopyranoside and acylated-α-chymotrypsins has been investigated. Both transformations are catalyzed by neighbouring nitrogen atom participation. The stereospecificity of acylated enzyme hydrolysis can be explained by the specific steric hindrance of nitrogen participation.

Introduction

In the early fifties, Fodor et al.[1,2] found that ethyl 3,4,6-tri-O-acetyl-β-D-glucopyranoside (**1**) was selectively deacetylated by alcoholysis· (Scheme 1.)



1 **2**

Scheme 1.

The reaction gave in very good yield ethyl 4,6-di-O-acetyl-2-deoxy-2-amino-β-D-glucopyranoside (**2**) containing the free C3-OH group. The product was well utilizable as an intermediate in the syntheses of amino mono[3]- and oligosaccharides[4]. In addition to its preparative use, the above reaction is noteworthy also from a theoretical point of view, as it can be regarded as the first solvolytic deacylation reaction with nitrogen-participation. The mechanism of this reaction has not been considered in details. Several years later Kupchan et al.[5] called attention to the participation of tertiary N atom in the solvolysis of cevadine orthoacetate diacetate. The reaction was interpreted as an appropiate model for esterase enzyme action.

Twenty years later after the first investigations, we gave a quantitative explanation of stereospecificity in the deacylation of acyl-α-chymotrypsin in terms of a free energy relationship[6].

A common feature of the above transformations is the assistance of N-atom located in a steric

position favourable to the O-acyl group. The primary purpose of this paper is to verify that the stereospecificity in hydrolysis of acylated-α-chymotrypsins is caused by the specific steric hindrance of N-atom participation. The secondary aim is to compare the N-assisted deacylation of 2-deoxy-2-aminoglucose and α-chymotrypsin derivatives.

Results and Discussion

One of the most widely studied enzymatic reactions is ester hydrolysis catalyzed by α-chymotrypsin. The rate of hydrolysis of esters containing an aryl group in β-position is by 1-2 orders of magnitude higher than that of esters containing aliphatic substituents. The reaction is highly stereospecific. The reaction mechanism was first elucidated by Gutfreund and Sturtevant[7] as illustrated below. (Scheme 2.)

$$\begin{array}{lcl} E + R{-}COOR' & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E.....R{-}COOR' \\ & & \downarrow k_2 \\ E + R{-}COOH & \xleftarrow{k_3} & E{-}O{-}\overset{\overset{O}{\|}}{C}{-}R + R'{-}OH \end{array}$$

if $R' = p{-}C_6H_4{-}NO_2$, then $k_2 >> k_3$.

Scheme 2.

In the case of active esters, e.g. 4-nitrophenyl ester, the k_2 value is much higher than k_3, hence the rate-determining step is the decomposition of the acyl-enzyme. The deacylation takes place with the participation of a heterocyclic nitrogen atom of histidine-57. The assistance of histidine-57 in the catalytic effect is promoted by aspartate-102 forming an ion pair with imidazolium ion [8,9] or a charge relay system[10,11] containing a H-bond between Asp-102 and His-57.

The steric structure of the active site of α-chymotrypsin was determined by X-ray diffraction[12]. The active site of the enzyme is shown schematically in Fig.1, where serine-195 is acylated by a substituted β-phenylpropionic acid.

The β-phenyl group is bound to the Niemann hydrophobic binding site[13] ρ^2. Owing to the crosslinkage formed by hydrophobic interaction and the covalent bond of the acylated serine-195 OH-group, the steric positions of the substituents of the acyl group are fixed. The R^2 substituent (τ) faces the inside of the biopolymer, while the R^1 substituent (μ) is oriented towards the solvent.

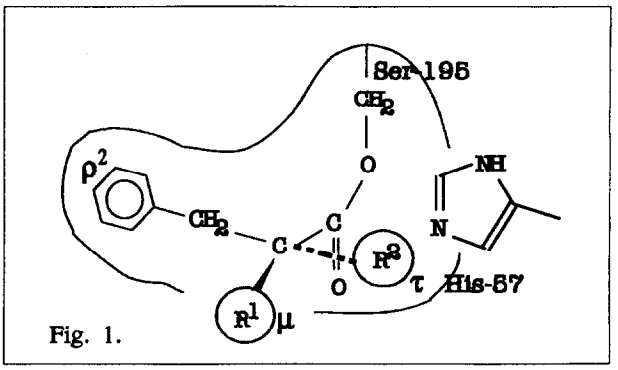


Fig. 1.

The R^1 group in position μ exerts no substantial steric effect, while the R^2 group in position τ depending on its size, hinders the assistance of N-atom in the heterocyclic base. The substituents R^1 and R^2 are in interchanged positions in substrates of R and S configurations, localized by cross-linkage.

In chiral substrates R^1 and R^2 groups exert different degrees of steric hindrance, and stereospecificity is due to this difference. In the acyl-enzyme involving S-α-methyl-β-phenylpropionate, (4) the

H atom is oriented towards the catalytic site of the enzyme, while in the case of R-α-methyl-β-phenylpropionyl-chymotrypsin (3) the CH_3 group is located in this position. (Fig.2.) The difference

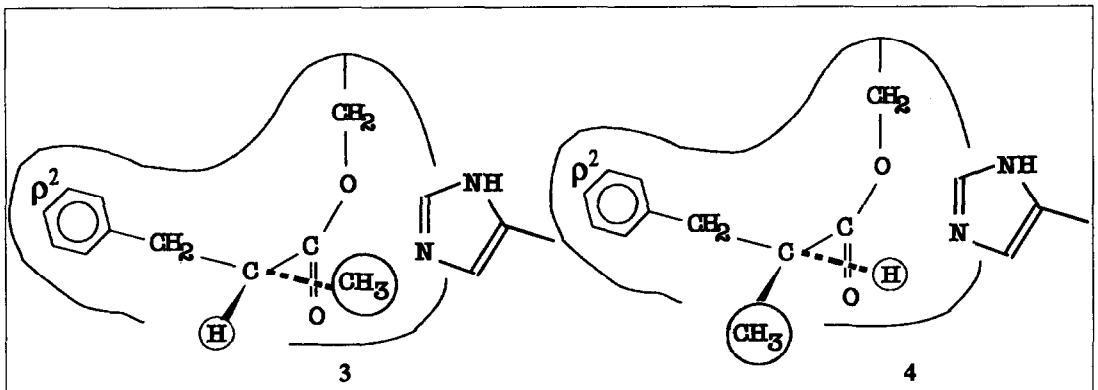


Fig 2.

in hydrolytic rates of the enantiomers is in accord with the difference in steric hindrance between the two substituents oriented towards the catalytic site (Table 1).

The validity of our assumption can be verified by the kinetic data of symmetrical substrates. Unsubstituted β-phenylpropionate has two H atoms in α-position, consequently, steric hindrance is expected to be the same as in case of the S-α-methyl-β-phenylpropionyl derivative. In the acyl-enzyme formed by the α,α-dimethyl-β-phenylpropionyl analog, the nucleophilic attack on the ester carbonyl group is hindered by $\alpha-$methyl group as in the case of R$-\alpha$-methyl-β-phenylpropionyl chymotrypsin, hence their reactivities are expected to be similar. These assumptions are fairly well supported by experimental data in Table 1.

Table 1. Deacylation constant (k_3) of acylated chymotrypsin

Substituent		
in τ position	in μ position	$k_3(s^{-1})$
H	H	1.9×10^{-1}
H	CH_3	1.2×10^{-1}
CH_3	H	4.1×10^{-3}
CH_3	CH_3	3.2×10^{-3}

The different steric hindrance caused by the different volumes of the substituents of enantiomeric substrates offers explanation for enzyme stereospecificity. This interpretation was applied for example, in the stereospecificity of dipeptide hydrolysis catalysed by aminoacylase[14].

Analysing the similirities in the deacylation of α-chymotrypsin and 2-deoxy-2-amino-glucopyranoside derivatives, we may draw the following conclusions. Both reactions occur by the participation of a N-atom located in a favourable steric position to the O-acyl group. This was proved by the X-ray diffraction data of compound 1 shown in Fig.3

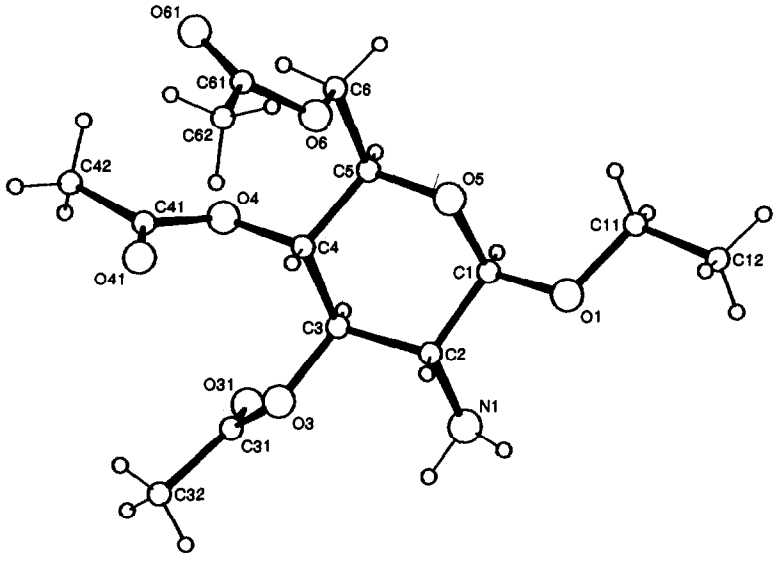


Fig. 3

The distances of atoms parcitipating in the solvolysys of the 3-O-acetyl groups in **1** are the folowing.

N1-O3 2.90 Å

N1-C31 3.61 Å

The distance beetween N^{ε} of His-57 and O^{δ} of Ser-195 in α-chymotrypsin is 3.0 Å according to Blow et al.[12] in good agreement with the above data.

Another argument for the similarity of enzymatic and non-enzymatic N-assistance is the comparable pKa values of the neighboring amino groups. The original experiments,[2] were carried out in alcoholic solution. Closer analogy can be found by reinvestigating the deacylation of ethyl 3,4,6-tri-O-acetyl-2-deoxy-2-aminoglucopyranoside in aqueous media. Study of the hydrolysis at pH 7.6 gave a rate more then sixty times higher than that of ethyl acetate pointing to catalytic deacylation in the former cas. N-assisted reactions require the presence of a free amino group. Determination of the pK value[16] of **1** gave pKa = 6.02, which is surprisingly low, although it can be accepted considering the pKa = 7.73 value of 2-amino-2-deoxy-D-glucose (literature data[15] 7.75). It is evident that at pH 7.6 ionization of the C2-NH_2 group allows N-participation in the hydrolysis. It should be noted that, according to GC-IR investigations, the reaction yields several products by O $\rightleftharpoons$ N and O $\rightleftharpoons$ O acetyl group migration.

The main difference between the deacylation of acetylated amino-sugar and acyl-α-chymotrypsin derivatives lies in the stereospecificity. The fixed conformation of the acyl moiety in enzymatic transformation ensures the specific steric hindrance discussed in this paper. There is no such effect in the case of glucosamine derivative. The structure and conformation of 2-deoxy-2-amino-D-glucopyranose theoretically allows the synthesis of an artificial enzyme model conformationally restricted by a C4-O derivative. We intend to study the above assumption by further investigations.

Experimental

Ethyl 2-deoxy-2-amino-β -D-glucopyranoside derivatives. Preparation of **1** and **2** was performed as described in the litterature[2]. The procedure resulted **2** which contained 10 % ethyl 3,6-di-O-acetyl-2-deoxy-2-amino-β-D-glucopyranoside by GC-IR analysis. After recrystallization of the mixture from isopropanol-diethyl ether (3:2) the purity of **2** was found to be higher than 98 % by GC . m.p.: 166 °C.

4-nitrophenyl esters. 4-nitrophenyl β-phenylpropionates were prepared by the dicyclohexylcarbodiimide method[17] as follows: 1,5 g (0,01 M) of the corresponding acid and 1,4 g (0,01 M) 4-nitro-phenol were dissolved in 30 ml ethylacetate. After chilling in a refrigerator 2,2 g (0,0105M) dicyclohexylcarbodiimide, dissolved in 10 mL ethylacetate, were added and the mixture was allowed to stand overnight in the refrigerator. After addition of 2 drops of acetic acid the crystalline dicyclohexylurea was filtered and the filtrate evaporated to dryness. The residue was recrystallised from ethanol. Compounds were characterized by their melting point, UV spectrum, and elementary analysis.

Compound 4-nitrophenyl propionate	m.p	Formula	Analysis			λmax (ethanol)
				calcd	found	
β-phenyl	99°C[x]	$C_{15}H_{13}NO_4$	C:	66.41	66.20	271 nm
			H:	4.82	4.50	
			N:	5.12	5.32	
α-methyl-β-phenyl	65°C	$C_{16}H_{15}NO_4$	C:	67.35	67.40	273 nm
			H:	5.30	5.45	
			N:	4.91	4.97	
S-α-methyl-β-phenyl	48°C	$C_{16}H_{15}NO_4$	C:	67.35	67.28	273 nm
			H:	5.30	5.40	
			N:	4.91	5.02	
α,α -dimethyl-β-phenyl	68°C	$C_{17}H_{17}NO_4$	C:	68.25	68.20	275 nm
			H:	5.72	5.80	
			N:	4.70	4.65	

[x] literature data 99 °C[18]

Table 2.

(S)-α-methyl-β-phenylpropionic acid was synthesized by enzymatic resolution of the methyl ester [19]. b.p. 100 °C/2Hg mm, $[\alpha]_D$ = +26.65 c = 3 benzene, (lit.[19] $[\alpha]_D$ = +27.06).

Infrared spectra were obtained on a Nicolet 170 SX-IR Spectrophotometer. GC-IR analyses were carried out with the same IR instrument connected on line with a Varian 3700 gaschromatograph using 30 m SPB-1 capillary column at 180-240 °C.

X-ray diffraction.

$C_{14}H_{23}O_8N$, a = 5.313/1/, b = 15.394/3/, c = 21.163/5/ Å, space group $P2_12_12_1$, X=ray data were collected on an Enraf-Nonius CAD4 diffractometer using Cu K_α radiation. Initial structure model was obtained by direct methods and refined by conventional full matrix leastsquares to a final R = 0.046 for 1145 reflexions with $F^2 > 3.0^*\sigma(F^2)$

Hydrolysis kinetics of **1**, **3** and ethyl acetate were studied by pH-stat measurement by the use of Radiometer pH-stat (TTT 80 Titrator, ABU 80 Autoburette, PHM 82 pH Meter, REC 80 Servograph)

The compound was dissolved in 20 mL destilled water and 0,1 n NaOH was added to the

solution up to pH 8.08. The consumption was registered at 25 °C for 20 hours. From these data pseudo first order k value was calculated: For compound **1** $k = 62\times10^{-6}$ sec^{-1} was obtained. Under identical conditions ethyl acetate resulted $k = 1{,}01\times10^{-6}$ sec^{-1} and **3** $k = 4{,}6\times10^{-6}$ sec^{-1}.

Enzymatic hydrolysis The hydrolysis of 4-nitrophenyl esters were investigated with UNICAM SP 8000 UV Spectrophotometer. 2,5 mL pH 7.5 phosphate buffer containing. 1×10^{-6} - 1×10^{-4}M α-chymotrypsin was thermostated at 25 °C in a UV spectrophotometer cuvette. To this solution 10-20 μL acetonitrile solution of 4-nitrophenyl β-phenylpropionate substrate was added with microseringe. Formation of 4-nitrophenol, registered at 400 nm, gave zero order rate constant. Under the experimental conditions k_{cat} values were equal with k_3, the rate constant of enzymatic deacylation.

The hydrolysis rate of the two enantiomers were very different and rate constants for enantiomeric species were calculated from measurements on racemic mixture.

The hydrolysis rate of (S)-α-methyl-β-phenylpropionic acid 4-nitrophenyl ester was measured separately and k3=1,4.10-1 sec.-1 was obtained which is in good agreement with the value obtained from racemic mixture.(Table 1)

Acknowledgement: We are indebted to Dr. M. Czugler and Prof. A. Kálmán for X-ray measurements and to Prof. S. Holly for GC-IR analysis.

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